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Membrane-bound receptor activator of NFκB ligand (RANKL) activity displayed by osteoblasts is differentially regulated by osteolytic factors

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ABSTRACT

Osteoclast formation is central to bone metabolism, occurring when myelomonocytic progenitors are stimulated by membrane-bound receptor activator of NFκB ligand (RANKL) on osteoblasts. Osteolytic hormones induce osteoblast RANKL expression, and reduce production of RANKL decoy receptor osteoprotegerin (OPG). However, rather than RANKL and OPG mRNA or protein levels, to measure hormonally-induced osteoclastogenic stimuli the net RANKL activity at the osteoblast surface needs to be determined. To estimate this we developed a cell reporter approach employing pre-osteoclast RAW264.7 cells transfected with luciferase reporter constructs controlled by NFκB (NFκB-RAW) or NFATc1 (NFAT-RAW)-binding promoter elements. Strong signals were induced in these cells by recombinant RANKL over 24 h. When NFκB-RAW cells were co-cultured on osteoblastic cells (primary osteoblasts or Kusa O cells) stimulated by osteolytic factors $1,25(OH)_2$ vitamin D_3 ($1,25(OH)_2D_3$) and prostaglandin E_2 (PGE_2), a strong dose dependent signal in NFκB-RAW cells was induced. These signals were completely blocked by soluble recombinant RANKL receptor, RANK.Fc. This osteoblastic RANKL activity was sustained for 3 days in Kusa O cells; with $1,25(OH)_2D_3$ withdrawal, RANKL-induced signal was still detectable 24 h later. However, conditioned medium from stimulated osteoblasts induced no signal. TGFβ treatment inhibited osteoclast formation supported by $1,25(OH)_2D_3$-treated Kusa O cells, and likewise blocked RANKL-dependent signals in NFAT-RAW co-cultured with these cells. These data indicate net RANKL stimulus at the osteoblast surface is increased by $1,25(OH)_2D_3$ and PGE_2, and suppressed by TGFβ, in line with their effects on RANKL mRNA levels. These results demonstrate the utility of this simple co-culture-based reporter assay for osteoblast RANKL activity.

1. Introduction

RANKL, a TNF-related protein, is a key factor regulating bone metabolism through its role in stimulating the formation and activation of bone resorbing osteoclasts [1]. The high bone mass and osteoclast deficiency in mice lacking RANKL or its receptor RANK [1–3] demonstrates the fundamental role of these molecules in bone metabolism. There is also ample clinical evidence that inhibiting RANKL actions significantly suppresses bone resorption both clinically and in mouse models [4,5]. The major source of RANKL at the bone surface is the bone forming cells, osteoblasts, as well as osteoblast-related osteocytes and bone lining cells. These cells produce both RANKL [6] and its secreted decoy receptor osteoprotegerin (OPG) in a highly regulated manner. Thus osteolytic factors like IL-6 family cytokines, PTH and dihydroxyvitamin D_3 ($1,25(OH)_3D_3$), drive osteoclast formation from hematopoietic cells by increasing RANKL production in osteoblasts [1,6] and reducing their production of OPG (illustrated in Fig. 1A). Activated T cells and myeloma cells also produce RANKL either in a secreted or shed soluble form [7,8]. Osteoblasts appear to produce only membrane-associated RANKL as, at least *in vitro*, contact between osteoclast progenitors and osteoblasts is required for osteoclast formation. However, soluble RANKL production by osteocytes has been suggested by recent work [9].

From the foregoing it is clear that measurement of RANKL in osteoblasts and stromal cells is of great interest. This is commonly studied by quantifying RANKL and OPG mRNA levels [6]. Estimating relative RANKL protein levels in osteoblast cell membrane extracts can be achieved by Western blotting but low sensitivity can be a problem and may also detect the significant amounts of RANKL bound to intracellular membranes [10,11]. Another concern is that it is not the RANKL levels *per se* that drive osteoclast formation but

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the net RANKL stimulus produced, i.e., net of OPG inhibitory effects [12]. The magnitude of this net RANKL stimulus can be inferred by studying osteoclast formation in co-cultures of bone marrow progenitors (or other hematopoietic population) with osteoblasts treated with stimuli of interest. However, such osteoclast formation is only observed after several days and involves complex differentiation pathway dynamics.

With these considerations we developed a simple, robust and rapid method to estimate functional RANKL stimulus in live osteoblastic cells by incubating osteoblasts with $RANK^+$ reporter cells derived from RAW264.7 cells. These cells efficiently form osteoclasts in response to RANKL, i.e., respond similarly to osteoclast precursors. RAW264.7 cells were stably transfected with vectors containing luciferase-expressing constructs under the control of promoters sensitive to NFκB and the NFATc1 transcription factors, both of which are critical for RANKL-dependent osteoclast formation [13,14]. Stimuli that induce signals in these reporter cells were confirmed as RANKL when their effects were ablated by soluble RANK.Fc, a truncated chimeric form of RANK which binds RANKL alone [3]. We used these methods to study two significant questions about RANKL induction – namely whether mRNA levels of RANKL stimulus reflects the net functional RANKL stimulus, and whether the induced RANKL stimulus is sustained or transient. The latter is particularly important in considering RANKL stimulation by relatively unstable local hormones like prostaglandins.

2. Materials and methods

2.1. Cells and reagents

Tissue culture medium used in all cultures was minimal essential medium-alpha (MEM) (Gibco BRL, Gaithersburg, MD) supplemented with fetal bovine serum 10% (CSL Biosciences, Parkville, Australia) and penicillin 50 U/mL; streptomycin 50 μg/mL and 2 mM L-glutamine (Gibco BRL).

C57BL/6J mice were obtained from Monash Animal Services, Clayton, Australia and were maintained at Monash Medical Centre Animal facility, Clayton, Australia with procedures approved by the institute ethics committee. Osteoblasts were prepared from newborn mouse calvariae by sequential digestion with 0.1% collagenase plus 0.2% dispase (Godo Shusei, Tokyo, Japan) and cultured for 3–4 days to confluence prior to use. Mouse bone marrow was obtained from adult (6–12 week old) male C57black6/J mice. Kusa O cells were cultured as previously described [15]. Rabbit anti-mouse/rat calcitonin receptor antibody was produced in house as per Tikellis et al. [16].

2.2. Cytokines and hormones

Recombinant GST-$RANKL^{158–316}$ (RANKL) was obtained from the Oriental Yeast Co. Ltd. (Itabashi City, Tokyo, Japan). Other recombinant proteins were obtained from R&D Systems (Minneapolis, MN). $1,25(OH)_2D_3$ was obtained from Wako Pure Chemical Co. (Osaka, Japan) and Prostaglandin E_2 (PGE_2) was obtained from Sigma Chemical Co. (Castle Hill, Australia). Other reagents were analytical grade obtained from Sigma unless noted.

2.3. Construction of stable reporter cell lines

NFκB-RAWs were generated as previously described [17]. Briefly, RAW264.7 cells were stably transfected with a G418-selectable vector containing a 3kB-Luc-SV40 luciferase reporter with a promoter containing NFκB binding sites (κB). Similarly, NFAT-RAWs were generated by stable transfection of RAW264.7 cells with a GL4.30 [*luc2P*/NFAT-RE/Hygro] (Promega, South Sydney, Australia) reporter construct with the luciferase production under the control of an NFAT-response element.

2.4. Luciferase reporter cell-based assays

NFκB-RAW and NFAT-RAW cells were routinely maintained in MEM/10%FBS. After trypsinisation 4×10^4 cells were seeded per well in tissue culture 96-wells (0.34 ml TPP grade, Thermo Fisher Scientific, Scoresby, Australia) and treatments were added as described. After incubation at 37 °C for 24 h, cells were rinsed in sterile PBS, lysed by the addition of 40 μl of passive lysis buffer (Promega) followed by an overnight incubation at 4 °C. Lysates were transferred to white 96 well plates (Thermo Fisher) for analysis with luciferase substrate (Promega) using an Envision (model 2103) multi-label plate reader (Perkin Elmer, Waltham, MA).

For luciferase reporter cell co-cultures with osteoblastic cells, the latter (Kusa O, primary osteoblasts and UMR106.01 cells) were seeded 2×10^4 cells/well in standard 96 well plates. Cytokine and hormone treatments were added to the cells at this point (unless otherwise noted) and incubated for 24 h. Reporter NFκB-RAW and NFAT-RAW cells (4×10^4 cells/well) were then added to these osteoblastic assays and the protocol followed the sequence mentioned as above.

2.5. Osteoclast formation assays

Kusa O cells were seeded in 10 mm diameter culture wells (2×10^4/well) in MEM/FBS, and mouse bone marrow cells (10^5 cells/well) added. $1,25(OH)_2D_3$ (10 nM) stimulation was added to the co-cultures, which were incubated at 37 °C for 3 days, then medium and mediators were replenished. After 7 days incubation cells were histochemically stained for TRAP as previously [18]. Some cell preparations were cultured on 6 mm diameter glass coverslips (Thermo Fisher), which were fixed in cold (−20 °C) acetone and immuno-stained with anti-calcitonin receptor (CTR) antibody (5 μg/ml) as previously [19].

2.6. Analysis of mRNA expression

Kusa O cells were cultured in six well plates with or without hormonal stimulation (MEM with 2%FBS). RNA was isolated using TRIZOL (Invitrogen) and treated with DNase (Ambion, Austin, TX) according to the manufacturer's instructions. cDNA was prepared from 5 μg RNA Superscript III Double-Stranded cDNA Synthesis Kit (Invitrogen). Real-Time PCR analysis (Stratagene Mx3000P, Agilent, Santa Clara, CA) was performed using SYBR Green mix (Invitrogen) according to manufacturer's instructions. Data were normalised to hypoxanthine-guanine phosphoribosyl transferase (HPRT)-1 and represented as fold induction over untreated time-point control. The oligonucleotide primer sequences (Sigma Genosys, Castle Hills, Australia) were as follows: RANKL (Gene Accession Number NM_011613), forward primer: 5′-TCCAGCTATGATGGAAGGCT-3′, reverse primer: 5′-GTACCAAGAGGACAGAGTG-3′. HPRT-1 (Gene Accession Number NM_013556), forward primer: 5′-TGATTAGCGATGATGAACCAG-3′, reverse primer: 5′-AGAGGGCCACAATGTGATG-3′.

2.7. Statistical analysis

Statistical analyses were performed using one way ANOVA with Tukey's *post hoc* test, using Graphpad Prism 5 software (Graphpad Software, San Diego, CA). In all cases, $P < 0.05$ was considered significant. All values are presented as mean ± standard error. Data in this manuscript was audited in accordance with the NHMRC (Australia) Code for the Responsible Conduct of Research (PHI Data Audit No. 11–29).

3. Results

3.1. Osteoblast RANKL regulation and the characterisation of NFκB-reporter cells

Kusa O is a pre-osteoblastic and pre-adipocytic cell line that supports osteoclast formation and forms mature osteoblasts in long-term culture [15]. RT-PCR analysis confirmed that 10 nM $1,25(OH)_2D_3$ treatment of Kusa O stromal cells enhanced RANKL mRNA (Fig. 1B). Consistent with this, Kusa O cells co-cultured with mouse bone marrow cells in the presence of $1,25(OH)_2D_3$ for 7 days resulted in osteoclast formation, evidenced by large numbers of $TRAP^+$ and CTR^+ mononucleated and multinucleated cells (Fig. 1C).

For NFκB-RAW cell characterisation, 4×10^4 cells were cultured per 6 mm diameter well for 24 h in the presence of recombinant RANKL. The cells showed a robust and dose dependent increase in luciferase signal with RANKL concentration that was maximal at 100 ng/ml, and blocked by 300 ng/ml recombinant RANK.Fc protein (Fig. 1D and E). Shorter periods of RANKL exposure similarly induced a signal but this was usually weaker and less robust (data not shown) so in further experiments, only 24 h incubations were used unless otherwise noted. Other NFκB inducing stimuli were also tested on these cells including TNF (20 ng/ml) (Fig. 1E) and LPS (100 ng/ml) (data not shown), which both induced a strong signal, but as expected these signals were not blocked by RANK.Fc. NFAT-RAW cells prepared and cultured similarly also exhibited a similar dose dependent increase to that observed in NFκB-RAW cells in response to RANKL stimulation (data not shown).

3.2. The use of reporter cells to measure membrane-bound RANKL stimulus in osteoblasts

To test the ability of Kusa O cells to make soluble RANKL detectable by the reporter cells, we incubated Kusa O cell cultures (initially 90% confluent) for 3 days in the presence or absence of $1,25(OH)_2D_3$. Conditioned medium (30%) from these cultures was added to NFκB-RAW cells for 24 h but no significant luciferase signal was observed (Fig. 2A) suggesting little or no transfer of activity RANKL. We then tested the response of NFκB-RAW cells in contact with Kusa O cells stimulated with $1,25(OH)_2D_3$ (illustrated in Fig. 2B). We first confirmed recombinant RANKL-induced signal in these cells was not affected by $1,25(OH)_2D_3$ (Fig. 2C). NFκB-RAW cells were then added to Kusa O cells pre-treated with $1,25(OH)_2D_3$ for 24 h, and a strong dose dependent signal was observed after 24 h of co-culture (Fig. 2D). A maximal stimulation was attained with 100nM $1,25(OH)_2D_3$. Signals induced by $1,25(OH)_2D_3$ were completely ablated by 300 ng/ml RANK.Fc (Fig. 2E) and for further studies this concentration of 300 ng/ml RANK.Fc was routinely used. Co-cultures with NFAT-RAW cells showed a similar pattern (data not shown) indicating that both reporter RAW cell types can be used to assess net RANKL stimulus in Kusa O cells. We also found that primary calvarial mouse osteoblasts also induced signals in reporter NFκB-RAW cells, when treated with the commonly used stimuli 10 nM $1,25(OH)_2D_3$ and 10^{-7} M PGE_2. (Fig. 2F); again this was ablated by the presence of RANK.Fc. Rat osteoblastic UMR106.01 cells also generated $1,25(OH)_2D_3$-dependent RANKL signals in co-culture (data not shown).

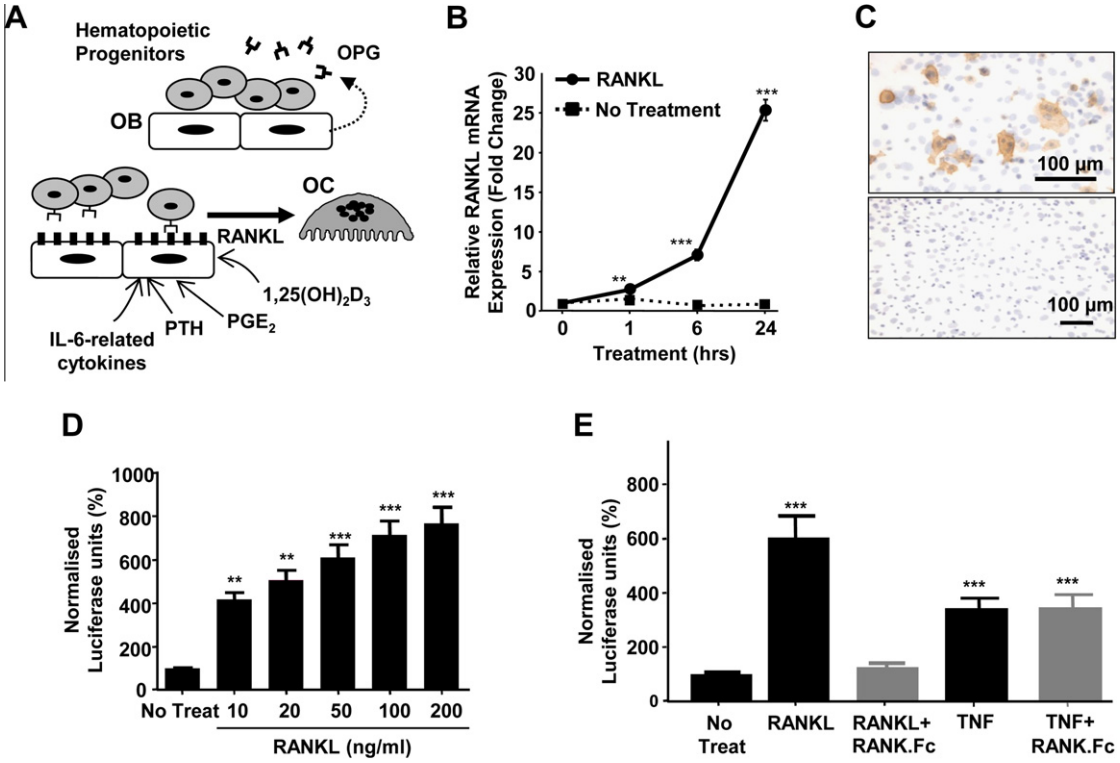

Fig. 1. RANKL production by osteoblastic cells and the characterisation of RANKL-elicited luciferase responses of NFκB-RAW cells. (A) Diagram illustrates osteoclast formation supported by hormonally-stimulated osteoblasts. Osteoblastic cells (OB) and progenitors in unstimulated state produce OPG (shown top). With the stimulation of PTH and osteolytic factors the osteoblasts produce RANKL (and less OPG), driving formation of osteoclasts (OC) (lower diagram). (B) Regulation of mRNA levels of RANKL in Kusa O osteoblastic cells stimulated by $1,25(OH)_2D_3$. (C) Confirmation of osteoclast formation in $1,25(OH)_2D_3$ treated bone marrow cells with Kusa O cells, showing CTR immunostaining (brown colour; top image) and immunostaining control (lower image); black bars = 100 µm. (D) Dose response of signal elicited in NFκB-RAW cells to recombinant RANKL stimulation. (E) Signals elicited in NFκB-RAWs with RANKL (100 ng/ml) but not TNF (20 ng/ml) were blocked by RANK.Fc (300 ng/ml). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

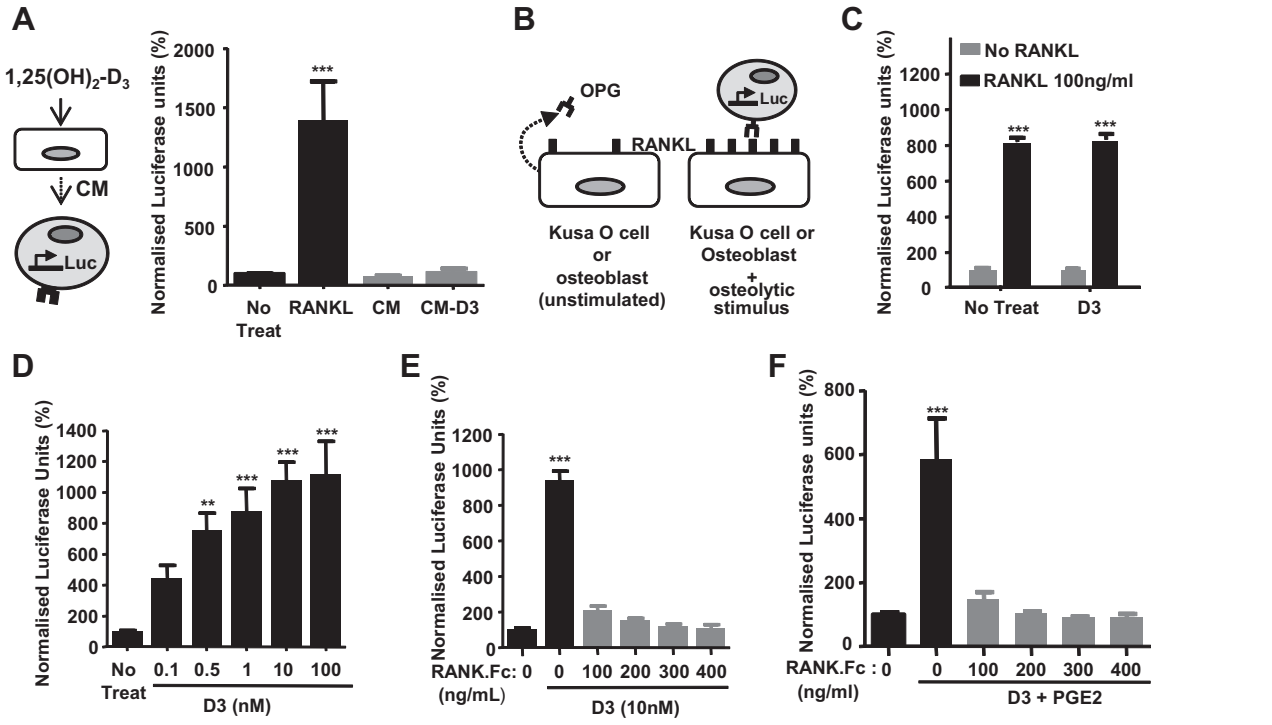

Fig. 2. The use of NFκB-RAW reporter cells to detect expressed RANKL protein in Kusa O and primary osteoblasts. (A) Lack of detectable soluble RANKL activity in 72 h conditioned medium from Kusa O cells (CM) and $1,25(OH)_2D_3$-stimulated Kusa O cell for (CM-D3); RANKL treatment (100 ng/ml) was employed as a positive control. Protocol is illustrated in diagram to the left. (B) Diagram illustrating the transfected reporter cell co-culture method employed in this study. (C) Lack of direct effects of $1,25(OH)_2D_3$ on signal induced in NFκB-RAWs. (D) Dose response of NFκB-RAW reporter cells to $1,25(OH)_2D_3$-stimulated in co-culture with Kusa O cells at 24 h. (E) RANK.Fc blocks elicited signals in NFκB-RAWs in co-culture with $1,25(OH)_2D_3$ stimulated Kusa O cells. (F) RANK.Fc similarly blocks signals induced in NFκB-RAWs by co-culture with primary osteoblasts stimulated by $1,25(OH)_2D_3$ plus PGE_2.

3.3. Persistence of RANKL activity in stimulated Kusa O cells

To determine the stability of the induced RANKL activity in stromal cells and to determine whether the protocol we adopted in Fig. 2 are the best options, we varied the time at which the stimulus (10^{-8} M $1,25(OH)_2D_3$) was added relative to the reporter cells (illustrated in Fig. 3A). 'Day 0' was taken as the time point at which reporter NFκB-RAW cells were added to the Kusa O cells; 'Day −1' is the day prior to NFκB-RAW addition, 'Day −2' 'Day −3' were 2 and 3 days prior. NFκB-RAWs were incubated in these co-cultures for 24 h (i.e., to 'Day +1) and then were lysed for luciferase analysis. We first varied the time course of stimulation in the following manner: no stimulus; $1,25(OH)_2D_3$ stimulus added at Day −1 then removed (washed out) at Day 0; $1,25(OH)_2D_3$ stimulus added at Day 0 only; $1,25(OH)_2D_3$ stimulus added at Day −1, then again at Day 0. In each case there was a full change of medium at Day 0 just prior to reporter cell addition. When $1,25(OH)_2D_3$ stimulation was added at Day −1 then washed out at Day 0 a diminished signal was observed compared to cultures where stimulus continued for a further 24 h (Fig. 3B). Nevertheless, this suggests that RANKL signal persists significantly even when the stimulus is removed. When $1,25(OH)_2D_3$ was added only with the reporter cells (i.e., at Day 0) the signal was detected but as might be expected this was weaker than that achieved by 24 h pre-stimulation. This is consistent with 24 h being required for RANKL protein expression to be maximal.

When $1,25(OH)_2D_3$ stimulus (10^{-8} M) was added at 3 or 2 days prior to reporter cell addition (Day 0), the signal obtained was equivalent to that observed when $1,25(OH)_2D_3$ was added 1 day prior (Fig. 3C). This suggests that either the $1,25(OH)_2D_3$ or the RANKL induction (or both) were stable over this period. We also tested PGE_2 (10 nM) applied in the same way, and indeed a weaker but similarly persistent signal was observed over this period.

3.4. Employing reporter cells to study RANKL suppression – the influence of TGFβ

We previously showed TGFβ treatment suppresses RANKL mRNA levels in primary osteoblasts [20] and reduces osteoclast formation in co-cultures. We first confirmed that in Kusa O cells TGFβ reduced RANKL mRNA levels induced by $1,25(OH)_2D_3$ (Fig. 4A). We then investigated the effects of TGFβ on levels of membrane RANKL activity in Kusa O cells using reporter cell/Kusa O co-cultures. However, first we tested the direct effects of TGFβ on our reporter cells and found TGFβ had a significant negative effect on luciferase signals in NFB-RAW cells (Fig. 4B; left panel). This observation suggested that this reporter cell was unsuitable for investigating TGFβ actions on Kusa O RANKL activity. In contrast, TGFβ (5 ng/ml) did not inhibit luciferase signals in NFAT-RAW cells, indeed enhanced them (Fig. 4B; right panel). Using NFAT-RAW cells co-cultured with $1,25(OH)_2D_3$-treated Kusa O we found that the strong RANKL-activity signal was indeed suppressed by TGFβ (Fig. 4C). TGFβ also blocked osteoclast formation in bone marrow/Kusa O co-cultures (Fig. 4D and E), but this inhibition was rescued by adding recombinant RANKL (200 ng/ml). This indicates that TGFβ inhibition of osteoclast formation is solely due the suppression of RANKL activity in Kusa O cells (Fig. 4D and E).

4. Discussion

This study has demonstrated that a simple co-culture method employing NFκB and NFAT sensitive reporter cells can be used to assess the levels of membrane-associated RANKL in osteoblastic cells. We succeeded in generating stably transfected reporter cells from pre-osteoclast/macrophage RAW264.7 cells (which are generally difficult to transfect) resulting in cells luciferase-responding

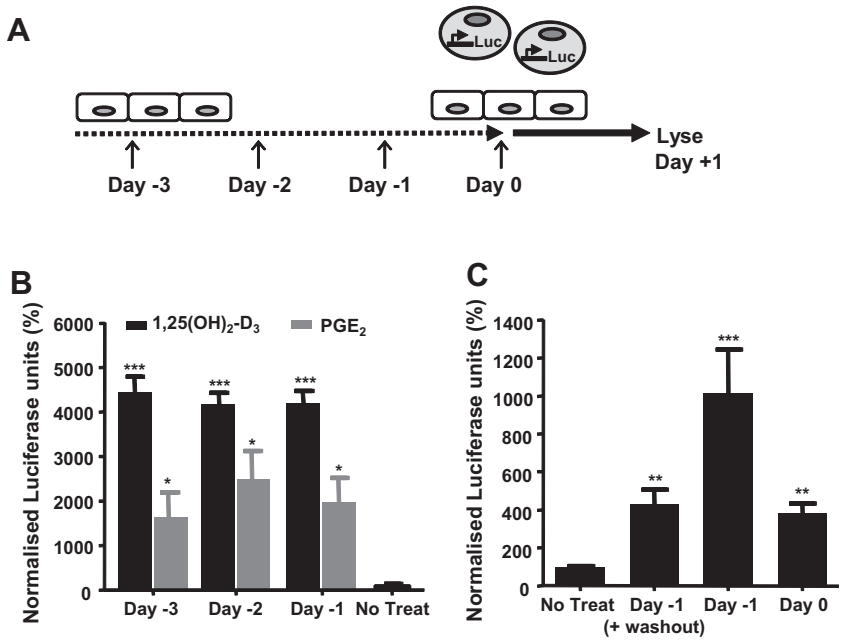

Fig. 3. Time course of signal induction in NFκB-RAWs co-cultured with Kusa O, and persistence of the RANKL induction by $1,25(OH)_2D_3$ and PGE_2. (A) Diagram illustrating the stimulation protocol – 'Day 0' was the point when NFκB-RAWs were added to Kusa O cells. Stimuli were added 1, 2 or 3 days prior (i.e., 'Day −1', Day −2' and 'Day −3', respectively) to Day 0. Cells were lysed for analysis 24 h after NFκB-RAWs addition ('Day +1'). (B) Effect of adding $1,25(OH)_2D_3$ stimulus on different days, as indicated. "Day −1 + washout" indicates that cells were treated on Day '−1' then the next day (Day 0) medium was replaced with medium without $1,25(OH)_2D_3$. (C) Persistence of the signal elicited by adding $1,25(OH)_2D_3$ (D3) and PGE_2 over 3, 2 or 1 days prior to reporter cell addition to the cultures.

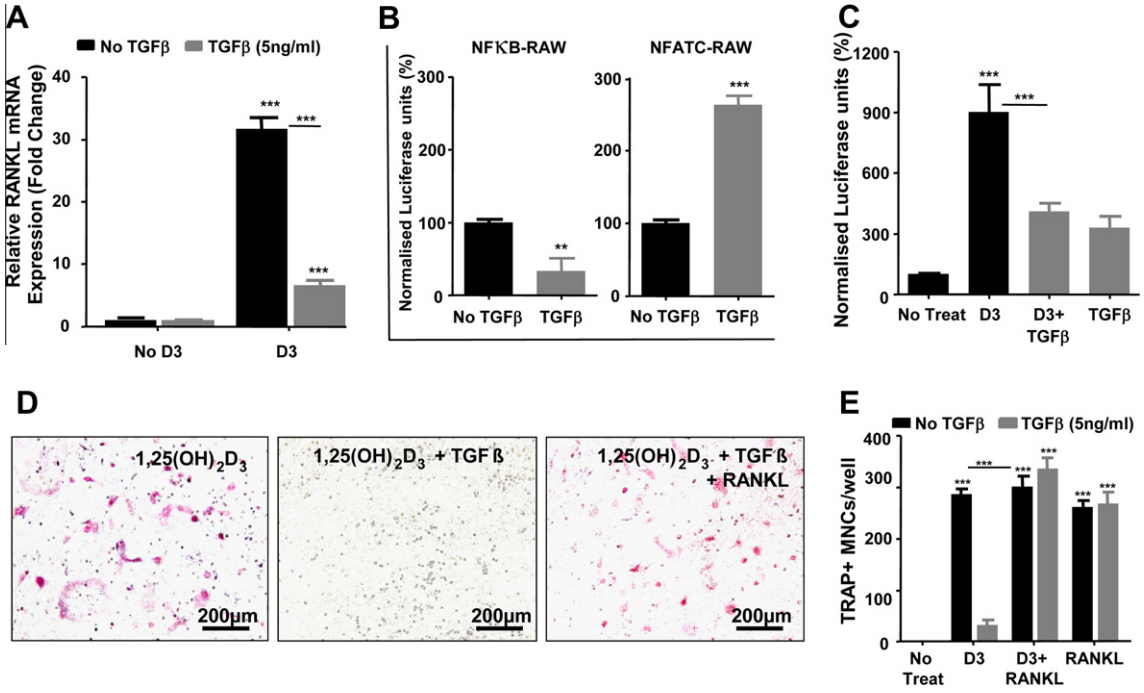

Fig. 4. TGFβ suppression of RANKL levels in Kusa O osteoblastic cells. (A) TGFβ suppressed $1,25(OH)_2D_3$-induced RANKL mRNA levels in Kusa O cells at 24 h. (B) NFκB-RAW cells treated with TGFβ showed reduction in signal (left) while NFAT-RAW cells treated with TGFβ showed increased signal (right). (C) NFAT-RAW cells co-cultured with $1,25(OH)_2D_3$-treated Kusa O cells showed signal reduction with TGFβ. (D) Images of $TRAP^+$ MNC formation in $1,25(OH)_2D_3$ treated bone marrow/Kusa O co-cultures. TGFβ inhibition of osteoclast formation was rescued by recombinant RANKL, black bars = 100 µm. (E) Quantitative data from co-cultures illustrated in D.

cells highly responsive to RANKL that were robustly stimulated when incubated in co-cultures with RANKL-expressing osteoblasts. This enabled a consistent and simple assay system for osteoblast RANKL activity, our results showing consistency with osteoblast RANKL mRNA expression levels and RANKL-dependent osteoclast formation. Our data indicates that relative quantification of functional RANKL signal strength on osteoblast membranes can be attempted in this way, as the results shown in Fig. 2D showed a clear dose response to increasing stimulus. Comparison of the magnitude of the RANKL-induced signals in Fig. 2E (Kusa O) and

Fig. 2F (osteoblasts) suggests that of the two the Kusa O cells provided a greater RANKL stimulus, however a careful estimate of the RANK.Fc concentration needed to ablate the RANKL signal would be needed to confirm this. This is because the linearity of the relationship between RANKL level and luciferase response is unclear, and indeed may be modified by other factors produced by the osteoblastic cells.

Measuring RANKL stimulus using a range of different assays is important to determine what elevates osteoclast formation in pathological tissues [21] and how osteoblastic cells participate in such osteolysis. It is particularly difficult with a membrane-bound protein to determine how much RANKL these cells deliver to osteoclasts, whether increased RANKL levels alone account for elevated osteoclast number. RANKL can be released in soluble form [22] and its detection in the circulation may indicate tissue RANKL levels, however, this may not give a useful indication of RANKL levels in bone, and it is unclear in some studies if detected circulating RANKL is bound to OPG. In our studies we were unable to find any transferable RANKL activity in conditioned medium from stimulated Kusa O (Fig. 2A) which suggests that at least under these conditions osteoblasts produce mainly cell-associated RANKL. Our data also suggests that RANKL activity induced by $1,25(OH)_2D_3$ and PGE_2 is surprisingly persistent long term (Fig. 3B) indicating these factors are stable under culture conditions as RANKL signal drops when the stimulus is removed (Fig. 3C). Alternatively, they may induce other autocrine RANKL-inducing factors from the stromal cells to sustain RANKL levels [23].

As well as investigating RANKL induction in osteoblasts we also sought to clarify paradoxical aspects of TGFβ action on osteoclast generation. TGFβ clearly enhances osteoclast progenitor responses to RANKL (and hence osteoclast numbers [20]) however when RANKL is delivered by osteoblasts TGFβ has inhibitory actions. Consistent with this, TGFβ reduced RANKL mRNA levels, but whether this abolishes RANKL protein production or induces production of some other inhibitor is unclear [20]. As TGFβ itself directly reduced luciferase signals in NFκB-RAW, cells we instead studied this phenomenon using NFAT-RAWs Kusa O co-cultures; these results indicated that RANKL activity induced by $1,25(OH)_2D_3$ was indeed abolished by TGFβ. It was also noted that TGFβ still induced NFAT signals which is significant as many osteoclast inhibitors diminish NFATc1 levels [14]. This data strongly suggests that RANKL activity is simply suppressed by TGFβ treatment, which also explains why simply adding recombinant RANKL was able to restore osteoclast formation in such co-cultures.

In summary, we have developed a reporter co-culture method to estimate the functional membrane-associated RANKL activity induced in osteoblastic cells by osteolytic factors. This demonstrated that RANKL activity is sustained until the osteolytic stimulus is removed, while TGFβ rapidly abolishes induced RANKL activity. This simple approach provides a useful and rapid method to study RANKL activity regulated by hormonal factors.

Acknowledgments

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